

Immunopathology and Infectious Diseases

Minor Histocompatibility Antigens Are Expressed in Syncytiotrophoblast and Trophoblast Debris

Implications for Maternal Alloreactivity to the Fetus

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The fetal semi-allograft can induce expansion and tolerance of antigen-specific maternal T and B cells through paternally inherited major histocompatibility complex and minor histocompatibility antigens (mHAg). The effects of these antigens have important consequences on the maternal immune system both during and long after pregnancy. Herein, we investigate the possibility that the placental syncytiotrophoblast and deported trophoblastic debris serve as sources of fetal mHAg. We mapped the expression of four mHAg (human mHAg 1, pumilio domain-containing protein KIAA0020, B-cell lymphoma 2-related protein A1, and ribosomal protein S4, Y linked) in the placenta. Each of these proteins was expressed in several placental cell types, including the syncytiotrophoblast. These antigens and two additional Y chromosome-encoded antigens [DEAD box polypeptide 3, Y linked (DDX3Y), and lysine demethylase 5D] were also identified by RT-PCR in the placenta, purified trophoblast cells, and cord blood cells. Finally, we used a proteomic approach to investigate the presence of mHAg in the syncytiotrophoblast and trophoblast debris shed from first-trimester placenta. By this method, four antigens (DDX3Y; ribosomal protein S4, Y linked; solute carrier 1A5; and signal sequence receptor 1) were found in the syncytiotrophoblast, and one antigen (DDX3Y) was found in shed trophoblast debris. The finding of mHAg in the placenta and in trophoblast debris provides the

first direct evidence that fetal antigens are present in debris shed from the human placenta. The data, thus, suggest a mechanism by which the maternal immune system is exposed to fetal alloantigens, possibly explaining the relationship between parity and graft-versus-host disease. (Am J Pathol 2012, 180:256–266; DOI: 10.1016/j.ajpath.2011.09.021)

During pregnancy, the maternal immune system must tolerate the semi-allograft of the fetus and its placenta. In human pregnancy, this is achieved through several means, including passively, through the highly restricted expression of class Ia and class II human leukocyte antigens (HLAs) in trophoblast cells. Active tolerance also plays an important role, for example, through trophoblast-associated immunoregulatory molecules.¹ Studies^{2–5} in mice and women indicate that there is an important role for regulatory T cells in immune tolerance to fetal antigens and, furthermore, that this tolerance can be established early in pregnancy.

Minor histocompatibility antigens (mHAg) represent a class of self-proteins encoded outside the major histocompatibility complex (MHC) that can elicit alloimmune responses across individuals. Many of these antigens are

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encoded by diallelic autosomal genes, others are encoded on the Y chromosome, and at least one gene is absent by deletion in some individuals.⁶ These antigens are HLA restricted: when broken down by antigen-presenting cells, one or more of the resulting peptides possess the correct structural properties to permit its presentation by class I or class II MHC molecules. Consequently, they are recognized by alloreactive CD4⁺ or CD8⁺ T cells from individuals lacking and, therefore, untolerized to, these proteins. mHAg were discovered as a cause of chronic graft rejection, graft-versus-host disease, and the graft-versus-leukemia effect in HLA-matched donor-recipient pairs.⁷⁻⁹

In addition to eliciting immune responses after transplantation, mHAg can also induce immune responses in the physiological context of pregnancy, in both mice and women. In murine pregnancy, CD4⁺ and CD8⁺ T cells are stimulated and tolerized by paternally inherited natural mHAg and transgenically expressed model minor antigens present in the seminal fluid and fetus.¹⁰⁻¹² These fetal antigen-specific T cells can be isolated from blood in multiparous mice and possess functional activity.¹³ The expansion and persistence of fetal antigen-specific cohorts of T cells in women long after pregnancy have also been recognized.¹⁴⁻¹⁶ The exposure of maternal T and B cells to fetal mHAg and the ensuing response are presumably tolerogenic in normal pregnancy but may have important clinical consequences. For example, multiparity has a negative impact on organ and hematopoietic stem cell transplantation, effectively increasing the risk of transplant rejection and graft-versus-host disease.¹⁷⁻¹⁹

Although the antigen-specific response of maternal lymphocytes to paternally inherited antigens during pregnancy is well documented, the route of exposure of these antigens remains uncertain. One possible route of exposure is fetal microchimerism, in which fetal and/or placental cells actively traffic across the placenta and lodge within maternal organs, where they remain long-lived.²⁰⁻²³ A second likely source of fetal antigen is the placenta. The human placenta, which possesses a hemochorial arrangement, is bathed by maternal blood during the latter two thirds of pregnancy. The syncytiotrophoblast forms the outer layer of the placental villi and is the major interface between fetal tissues and maternal blood. An abundance of placental material is shed into the maternal circulation, and this physiological property of the placenta is thought to have important consequences on maternal physiological and pathological responses in pregnancy.^{24,25} The terminally differentiated syncytiotrophoblast is continuously renewed by the fusion of underlying cytotrophoblast precursors, whereas aged or damaged portions of the syncytiotrophoblast layer are extruded into the maternal blood as large multinucleated structures called syncytial knots.²⁵ Other trophoblast debris shed into the maternal circulation includes mononuclear cytotrophoblasts and subcellular microparticles and nanoparticles.²⁶⁻²⁸ Gram quantities of trophoblastic elements may be deported from the placenta each day in normal pregnancy,²⁹⁻³² and the quantity of deported material may substantially increase during preeclamptic pregnancy.^{33,34} This trophoblast debris is rapidly cleared from the maternal circulation without

apparently generating an inflammatory immune response in normal pregnancy.³¹

Researchers^{25,35-37} have proposed that the shedding of trophoblast debris from the placenta provides an important avenue for immunological exposure of the mother to paternally derived fetal antigens, serving to establish maternal immune tolerance to the fetus. Whether such tolerance of the maternal adaptive immune system is possible is unclear, however, because the syncytiotrophoblast and villous cytotrophoblasts lack polymorphic HLA. The objective of this study, therefore, was to investigate whether mHAg are present in the human placenta and in trophoblast debris shed from the placenta. We chose to analyze the expression of six minor antigens in the placenta, whose properties and mRNA expression pattern are summarized in Table 1: the autosomal antigens [human mHAg 1 (HMHA1), KIAA0020, and B-cell lymphoma 2-related protein A1 (BCL2A1)]; and the Y chromosome-encoded antigens [lysine demethylase 5D (KDM5D), ribosomal protein S4, Y linked (RPS4Y1), and DEAD box polypeptide 3, Y linked (DDX3Y)]. The HMHA1 and KDM5D antigens elicit a maternal response in pregnancy,^{13,15,16} and, like KDM5D, RPS4Y1 and DDX3Y were of interest as male antigens. BCL2A1 expression was explored because it is a regulator of apoptosis, a property that may be of significance given the putative role of apoptosis in trophoblast shedding.^{28,30} Finally, KIAA0020 is of interest as a potentially novel inducer of the maternal immune system. By using molecular, immunohistochemical (IHC), and proteomic approaches, we provide the first evidence that the human placenta is a rich source of these and other fetally derived mHAg.

Materials and Methods

Tissue Procurement

All tissues were obtained in accordance with protocols approved by the University of Kansas Medical Center Institutional Review Board (Kansas City, KS) and the Auckland Regional Ethics Committee (Auckland, New Zealand). First- and second-trimester placentas (8 to 12 and 13 to 21 weeks gestational age, respectively) were obtained from elective terminations of normal pregnancies from a Kansas City-area private office (Overland Park, KS), the University of Chicago Department of Obstetrics and Gynecology (Chicago, IL), the Epsom Day Unit, Greenlane Clinical Center (Auckland, New Zealand) and the Auckland Medical Aid Clinic (Auckland, New Zealand). Term placentas (≥ 37 weeks' gestational age) were obtained from the University of Kansas Hospital (Kansas City, KS) from uncomplicated pregnancies after elective cesarean sections in the absence of labor. Amniochorion membrane and placental sections were dissected from term placentas, and cord blood was collected after placental delivery.

IHC Data

Samples of first-trimester ($n = 5$ to 8 per antibody), second-trimester ($n = 2$ to 3 per antibody), and term ($n = 8$ to 10 per antibody) placentas, the latter with an attached basal plate (BP), and extraplacental membranes ($n = 6$ to 8) were fixed in 4% paraformaldehyde overnight, dehydrated through a graded series of ethanol, and embedded in paraffin. Sections (10 μm thick) were placed onto slides, rehydrated, and stained as previously described,⁵⁰ with modifications dependent on each antibody, as described later. For all antibodies, tissues were subjected to hot citrate antigen retrieval using Reveal buffer (BioCare Medical, Walnut Creek, CA). Nonspecific antibody binding was blocked in 10% goat serum (Sigma-Aldrich, St. Louis, MO). The rabbit polyclonal primary antibodies targeting HMHA1 (12 $\mu\text{g/mL}$), KIAA0020 (2 $\mu\text{g/mL}$), and RPS4Y1 (12 $\mu\text{g/mL}$), all obtained from Sigma-Aldrich, were added to tissue sections, which were then incubated overnight at 4°C. For slides incubated with anti-KIAA0020 antibody, tissue sections were permeabilized in 0.5% Tween 20 (Sigma-Aldrich) in PBS for 30 minutes at room temperature before blocking, and 0.05% Tween 20 was added to PBS for all wash steps. For slides incubated with rabbit anti-BCL2A1 (6 $\mu\text{g/mL}$; Epitomics, Burlingame, CA), antibody was added for 1 hour at room temperature. After the addition of biotinylated goat anti-rabbit secondary antibodies and the depletion of endogenous peroxidases, binding of primary antibody was detected using streptavidin-peroxidase and aminoethyl carbazole (Invitrogen, Carlsbad, CA).

Purification of Cord Blood Mononuclear Cells and Cytotrophoblast Cells

For isolation of cord blood mononuclear cells, blood was diluted 1:2 in PBS and layered over Histopaque 1077 (Sigma-Aldrich). The blood was centrifuged for 30 min-

utes at $400 \times g$, and cells at the serum-Histopaque interface were collected, washed, and counted by trypan dye exclusion, as previously described.⁵¹

Cytotrophoblast cells were isolated as previously described.⁵¹ Approximately 40 g of villous material was dissected from term placentas, rinsed with 0.9% saline, and subjected to enzymatic digestion using trypsin (Invitrogen) and DNase (Sigma-Aldrich). Dispersed cells were layered over fetal bovine serum and centrifuged at $1000 \times g$, and after washing, the pellet was resuspended in HBSS, layered over a 5% to 70% stepwise Percoll gradient (Sigma-Aldrich), and centrifuged at $1200 \times g$. The band corresponding to trophoblast cells was aspirated, and the cells were subjected to a second stage of purification using immunomagnetic beads (Miltenyi Biotec, Boston, MA) coated with anti-class I MHC (W6/32), as previously described.⁵¹ Cell purity was assessed using anti-cytokeratin 7 by IHC or flow cytometry and was determined to be >90%.

RNA Isolation and RT-PCR

RNA was extracted from whole placenta ($n = 3$ female and 5 male), purified cytotrophoblast cells ($n = 2$ female and 3 male), and cord blood mononuclear cells ($n = 2$ female and 2 male) using TRI Reagent (Applied Biosystems, Foster City, CA). RNA concentrations were quantified using a BioPhotometer (Eppendorf, Hauppauge, NY), and 1 μg of RNA was reverse transcribed using Superscript III Reverse Transcriptase and random primers (Invitrogen) in a reaction volume of 20 μL . Parallel reactions containing RNA but no reverse transcriptase were included to verify the absence of genomic DNA in samples in the subsequent PCR steps (Fermentas, Glen Burnie, MD). Conventional RT-PCR was performed for DDX3Y, RPS4Y1, and glyceraldehyde-3-phosphate dehydrogenase, with primers designed using PrimerBLAST (National Center for Biotechnology Information, Bethesda,

Table 1. Properties and Expression of Human mHAGs

Minor antigen	Gene	Chromosome	HLA restriction	Function	Tissue expression (mRNA)	Reference no.
HA-1	<i>HMHA1</i>	19p13.3	HLA-A*0201, HLA-A*0206, HLA-B*60, and HLA-B*40012	GTPase activator (putative)	Hematopoietic cells	38–40
HA-8	<i>KIAA0020</i>	9p24.2	HLA-A*0201	RNA-binding protein (putative)	Broad	41
ACC-1 and ACC-2	<i>BCL2A1</i>	15q24.3	HLA-A*2402 and HLA-B*4403	Negative regulator of apoptosis	Hematopoietic cells	42
SLC1A5	<i>SLC1A5</i>	19q13.3	HLA-B*4002	Amino acid transporter	Some carcinomas, especially colorectal	43 and 44
LB-SSR1-1S	<i>SSR1</i>	6p24.3	HLA-A*0201	Signal sequence receptor in endoplasmic reticulum	Hematopoietic cells, small intestine	44 and 45
A2/HY and B7/HY	<i>KDM5D</i>	Yq11	HLA-A*0201 and HLA-B*2705	Protein- containing zinc finger domains	Broad; high in hematopoietic cells	44 and 46
B52/HY and DRB3/HY	<i>RPS4Y1</i>	Yp11.3	HLA-B*5201 and DRB3*0301	RNA-binding protein	Broad	47 and 48
DQ5/HY and DRB1/HY	<i>DDX3Y</i>	Yq11a	DQB1*05 and DRB1*1501	RNA helicase, involved in spermatogenesis	Broad; high in testis	49

KIAA0020, pumilio domain-containing protein KIAA0020; *SLC1A5*, solute carrier 1A5; *SSR1*, signal sequence receptor 1.

MD) and synthesized by Integrated DNA Technologies (Coralville, IA). Primer sequences were as follows: DDX3Y forward, 5'-TGCTGTCGTATAGCTGTGGG-3'; DDX3Y reverse, 5'-TCTCGAGACCCAAACTGCT-3'; RPS4Y1 forward, 5'-AGATTCTCTTCCGTCGCAGA-3'; RPS4Y1 reverse, 5'-CATAGACCAGCGGAAATGT-3'; glyceraldehyde-3-phosphate dehydrogenase forward, 5'-ACCACACTCCATGCCATCAC-3'; and glyceraldehyde-3-phosphate dehydrogenase reverse, 5'-TCCAC-CACCCTGTTGCTGTA-3'. The RT reaction sample, 2 μ L, was subjected to PCR (Invitrogen) using one times PCR buffer, 0.2 mmol/L deoxyribonucleotide triphosphate, 1.5 mmol/L $MgCl_2$, 1.5 U Taq polymerase, and 5 mmol/L of each primer in a volume of 20 μ L. The PCR was performed with the following protocol: 94°C for 3 minutes; 35 cycles of 94°C for 45 seconds, 63°C for 30 seconds, and 72°C for 90 seconds; and 72°C for 5 minutes. TaqMan Gene Expression Assay kits for real-time RT-PCR were obtained from Applied Biosystems (Foster City, CA) (HMHA1, Hs00299628_m1; KIAA0020, Hs00972109_m1; BCL2A1, Hs00187845_m1; KDM5D, Hs01104415_m1; and β -actin, Hs99999903) and run according to the manufacturer's protocol. All RT-PCR samples were analyzed in triplicate, and a mean was generated from the three C_T values for each sample. For each individual placenta, purified trophoblast or cord blood sample, the mean for each mHAg was subtracted from the mean for β -actin to provide an mHAg C_T value that had been normalized to β -actin to account for discrepancies in RNA concentration or quality. These means were then pooled to generate an average C_T value for each antigen in each tissue type.

Protein Extraction

For analysis of protein content of shed trophoblast debris, an established *in vitro* model of trophoblast shedding⁵² was used, together with a proteomic approach. Trophoblast debris was harvested from 12 first-trimester placentas (8 to 12 weeks' gestational age). Placental tissue was washed with PBS (pH 7.4), dissected into approximately 400 mg of wet-weight explants, and transferred into Netwell inserts (Corning, Lowell, MA) placed in a 12-well culture plate (Becton Dickinson, Franklin Lakes, NJ). The explants were cultured in 3 mL of Dulbecco's modified Eagle's medium/F-12 medium (Invitrogen) containing 5% fetal bovine serum and 1% penicillin-streptomycin for 12 hours at 37°C, in a humidified atmosphere containing 5% CO_2 and 95% ambient oxygen. Trophoblast debris shed from the explants was collected from the bottom of the culture plate, and red blood cells were depleted by hypotonic lysis, while contaminating leukocytes were removed using anti-CD45-coated magnetic beads, as previously described.⁵² The trophoblast debris was subjected to two independent protein extraction methods to enrich the plasma membrane components in the extracts: Ludox CL cationic colloidal silica (Sigma-Aldrich) and direct trypsinization. The colloidal silica extraction protocol followed the method of Robinson et al.,⁵³ with minor modifications. For direct trypsinization, briefly, the trophoblast debris was suspended in 30 μ L of pre-

warmed 50 mmol/L NH_4HCO_3 containing 1 μ L of benzonase nuclease (Merck, Darmstadt, Germany). Sequencing-grade trypsin (Promega Corporation, Madison, WI), 1 μ L, was added and incubated at 37°C for 80 minutes, with 1 μ L of trypsin added at 20-minute intervals. The sample was centrifuged at $17,000 \times g$ at 4°C for 15 minutes, and the supernatant was collected.

To characterize proteins from the syncytiotrophoblast, protein extraction was performed directly on six placentas after extensive washing in PBS. Syncytiotrophoblast proteins were isolated from the surface of placental explants using extraction methods to enrich the plasma membrane component in the extract. Two independent methods were used to extract proteins: Ludox CL cationic colloidal silica (previously described) and Triton X-114 detergent (Sigma-Aldrich). The Triton X-114 extraction protocol followed the method of Dickerson et al.,⁵⁴ with minor modifications. To confirm enrichment of plasma membrane components in the trophoblast debris, the relative proportion of proteins found primarily in the plasma membrane was compared between a non-plasma membrane enrichment technique (radioimmuno-precipitation assay lysis) and the two plasma membrane enrichment techniques, Ludox CL cationic colloidal silica and direct trypsinization. Data were analyzed through the use of Ingenuity Pathways Analysis (Ingenuity Systems, Redwood City, CA) and showed a relative enrichment of 12% (O.J.H. and L.W.C., unpublished data).

MS Data

Liquid chromatography/tandem mass spectrometry (MS/MS) was used to sequence tryptic peptides in the extracts from which protein identities were determined. Samples were electrophoresed on a 4% to 15% SDS polyacrylamide gel, and either the entire lane was excised as a whole or the lane was excised in sections for MS analysis. The excised protein lanes were subjected to in-gel digestion with trypsin using a robotic workstation for automated protein digestion (DigestPro Msi; Intavis AG, Cologne, Germany), following the method of Shevchenko et al.⁵⁵ The MS measurements were made on the LTQ-Orbitrap hybrid MS (Thermo Scientific, Lafayette, CO). Extracted MS/MS spectra were automatically assigned to the best-matching peptide sequence using the SEQUEST algorithm and the Sequest Browser software package (ThermoFinnigan, Lafayette, CO). The false-discovery rate for the data was calculated by running a decoy search against the reversed Human Reference Sequence database (National Center for Biotechnology Information) using the same search parameters as the main analysis. The reversed database was built from the same copy of the Human Reference Sequence database that has been used for the main searches using the Decoy Database Tool (*Trans-Proteomic Pipeline*; Seattle Proteome Center, Institute for Systems Biology, Seattle, WA).

Results

RNA for mHAg in the Placenta and Cord Blood

To determine whether the placenta or fetal blood cells could serve as sources of mHAg, whole placental lysates, purified cytotrophoblast cells, and fetal cord blood RNA were examined by real-time RT-PCR for the presence of four genes known to encode mHAg proteins. The expression of mRNA for HMHA1, KIAA0020, BCL2A1, and KDM5D was found in placental lysates, purified cytotrophoblast cells, and fetal cord blood (Figure 1A). No expression of the Y chromosome–encoded mHAg KDM5D was found in samples from pregnancies with a female fetus. Because of lack of availability of TaqMan real-time PCR gene expression assay kits that distinguish between the male and female isoforms of the *DDX3Y* (*DDX3X*) and *RPS4Y1* (*RPS4X*) genes, we designed primers for conventional RT-PCR that would distinguish these transcripts. Like *KDM5D*, *DDX3Y* and *RPS4Y1* were identified in whole placenta, cytotrophoblast cells, and cord blood from male, but not female, fetuses (Figure 1, B and C).

Analysis of mHAg Proteins in the Placenta

General Properties of mHAg Immunoreactivities in the Placenta

First-trimester, second-trimester, and term placental villi, as well as term extraplacental membranes, were examined by IHC using antibodies targeting the mHAg proteins HMHA1, KIAA0020, BCL2A1, and RPS4Y1. All four minor antigens could be observed in both villous and extravillous trophoblast (EVT) populations, with variation occurring within and between placental samples, across gestation, and according to antigen and cell type (Figure 2 and Figure 3). IHC for *DDX3Y* and *KDM5D* was attempted using commercially available antibodies, but failed to yield any positive staining under a variety of conditions, including positive control testis tissue.

HMHA1 and RPS4Y1 expression was cytoplasmic (Figure 2, A, B, G, and H, and Figure 3, A, B, G, and H). KIAA0020 and BCL2A1 were also usually seen in the cytoplasm, but this was frequently accompanied by nuclear expression (Figure 2, C–F, and Figure 3, C–F). The RPS4Y1 antibody stained trophoblast cells (all subpopulations) of both male and female placentas, because this antibody does not distinguish between the Y- and X-encoded isoforms of this protein. Expression observed using this antibody is, therefore, described as RPS4X/Y1 hereafter.

Villous Trophoblast Cells

In the syncytiotrophoblast, immunoreactivity for all four mHAg examined was observed, although staining intensity for each antigen always varied, both within and between placentas. Immunoreactivity for BCL2A1 was generally stronger and more uniform in first- and second-trimester villous cytotrophoblasts and syncytiotrophoblasts than at term (Figure 2, E and F, and Figure 3E). However, in three of

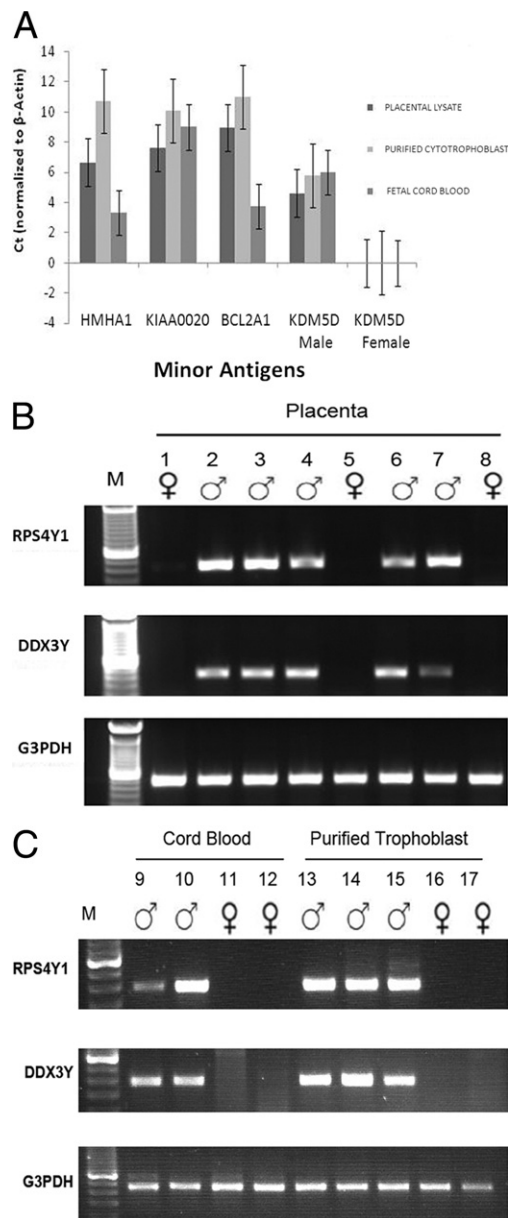


Figure 1. Minor antigen mRNA expression in placenta, cytotrophoblast cells, and fetal cord blood. **A:** Real-time RT-PCR for autosomally encoded mHAg (HMHA1, KIAA0020, and BCL2A1) and the Y chromosome–encoded mHAg (*KDM5D*), for placental lysate and purified cell populations. Data are expressed as the mean \pm SEM difference in C_T values between the gene of interest and β -actin for the same samples, calculated as described in *Materials and Methods*. The average \pm SEM β -actin values in each sample were as follows: 24.9 ± 0.59 (placenta), 21.4 ± 0.24 (cytotrophoblast), and 26.53 ± 1.56 (cord blood mononuclear cells). **B** and **C:** Conventional RT-PCR data for placental lysate and purified cells, respectively. Glyceraldehyde 3-phosphate dehydrogenase (*G3PDH*) was used as a control; the sex of the infant is indicated by symbols above each lane. CB, cord blood mononuclear cells; CT, cytotrophoblast cells; M, mol. wt. marker.

the eight term placentas examined, BCL2A1 was often concentrated or restricted to areas of aggregated syncytiotrophoblast nuclei, possibly representing syncytial knots before their shedding from the placenta.

KIAA0020 and RPS4X/Y1 proteins were observed in villous trophoblasts of all placentas throughout gestation. Immunoreactivity varied within and between placentas (Figure 2, C, D, G, and H, and Figure 3, C, D, and G).

KIAA0020 was always found in the cytoplasm, and some first-trimester samples exhibited nuclear staining as well. This protein was often concentrated at the microvillous surface and displayed stronger immunolocalization in the syncytiotrophoblast than in the cytotrophoblast.

HMHA1 immunoreactivity in the villous trophoblast was only observed consistently in first-trimester samples. One of five second-trimester samples, and none of the term samples, expressed this protein in the syncytiotrophoblast (Figure 2, A and B, and Figure 3A). Within each individual first-trimester sample, trophoblast expression of HMHA1 was variable. In the first- and second-trimester samples, syncytial immunoreactivity was always stronger than cytotrophoblast staining.

EVT Data

The expression of all four mHAg proteins was frequently observed in EVTs. BCL2A1 expression was moderate in first-trimester (Figure 2E) and second-trimester (data not shown) trophoblastic columns, whereas at term, staining for BCL2A1 was weak or absent in the EVTs, basal plate, and chorion membrane (Figure 3F). KIAA0020, HMHA1, and RPS4Y1

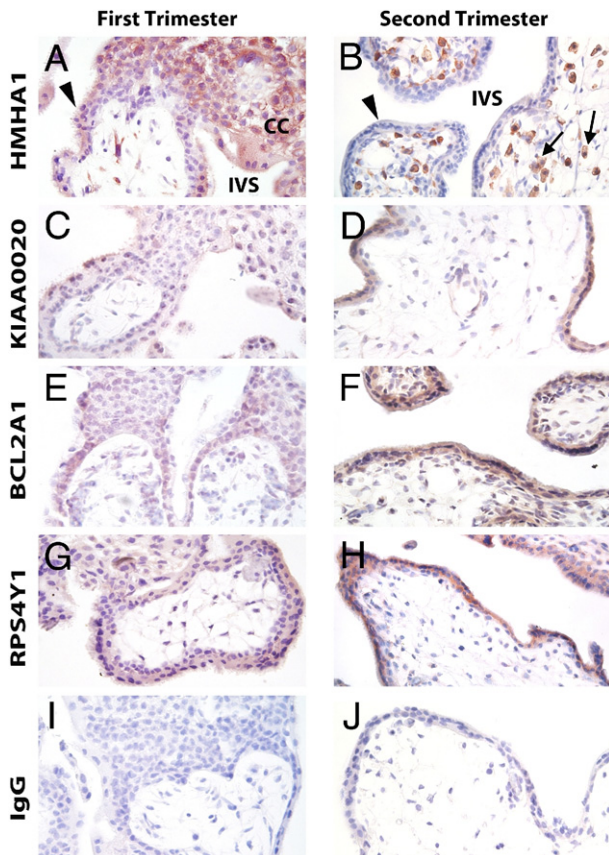


Figure 2. mHAg expression in first- and second-trimester placentas. **A, C, E, G, and I:** First-trimester placenta (gestational age, 8 to 12 weeks). **B, D, F, H, and J:** Second-trimester placenta (gestational age, 13 to 21 weeks). Reddish-brown staining represents specific immunoreactivity of mHAg-specific antibodies with cells; bluish-purple staining, hematoxylin counterstain. **Arrowheads** indicate syncytiotrophoblast; **arrows**, Hofbauer cells. IVS, intervillous space; CC, trophoblast cell column.

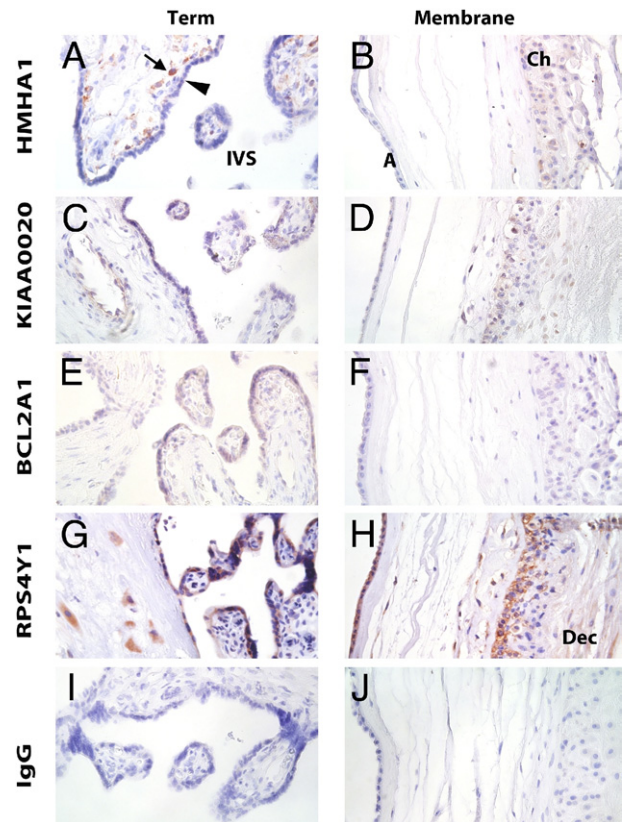


Figure 3. mHAg expression in term placentas and extraplacental membranes. **A, C, E, G, and I:** Term placenta. **B, D, F, H, and J:** Extraplacental membranes. **Arrowhead** indicates syncytiotrophoblast; **arrow**, Hofbauer cells. A, amnion membrane; Ch, chorion membrane; Dec, decidua; IVS, intervillous space.

antibodies consistently reacted with EVT cells throughout gestation; in the term chorion membrane, trophoblast cells proximal to the amnion exhibited stronger immunoreactivity for HMHA1 and RPS4Y1 than those distal to the amnion (Figure 3, B and H).

Fetal Leukocytes

Fetal leukocytes contained within the vasculature of the term placentas were generally negative for KIAA0020, BCL2A1, and RPS4X/Y1; however, a few weakly positive leukocytes were occasionally present in these samples. Fetal leukocytes in term placentas consistently and strongly expressed HMHA1 (data not shown).

Hofbauer and Mesenchymal Cells

Hofbauer cells, which are found within the villous mesenchyme and share lineage with macrophages, showed strong expression of HMHA1 throughout gestation (Figure 2, A and B, and Figure 3A). In the term placenta, stromal fibroblasts could also be seen to express HMHA1. BCL2A1, KIAA0020, and RPS4X/Y1 antibodies generally did not react with placental fibroblasts or Hofbauer cells, with the exception that most second-trimester placentas exhibited weak staining of both cell types.

Table 2. Antigenic Minor Histocompatibility Proteins Detected by MS in Shed Trophoblast Debris and in the Syncytiotrophoblast

mHAg	Protein name	Gene	Trophoblast debris	Syncytiotrophoblast
DRB1/HY and DQ5/HY	ATP-dependent RNA helicase DDX3Y	<i>DDX3Y</i>	+	+
B52/HY and DRB3/HY	40S ribosomal protein S4, Y isoform 1	<i>RPS4Y1</i>	—	+
SLC1A5	Neutral amino acid transporter B(0) isoform 1	<i>SLC1A5</i>	—	+
LB-SSR1-1S	Translocon-associated protein subunit α	<i>SSR1</i>	—	+
HA-1	Minor histocompatibility protein HA-1	<i>HMHA1</i>	—	—
HA-8	Pumilio domain-containing protein KIAA0020	<i>KIAA0020</i>	—	—
ACC-1 and ACC-2	Bcl-2-related protein A1	<i>BCL2A1</i>	—	—
A2/HY and B7/HY	Lysine-specific demethylase 5D	<i>KDM5D</i>	—	—

The protein name and gene symbol of each minor antigen are given.

KIAA0020, pumilio domain-containing protein KIAA0020; *SLC1A5*, solute carrier 1A5; *SSR1*, signal sequence receptor 1; +, found; —, not found.

Endothelial cells were consistently immunoreactive with the KIAA0020 antibody throughout gestation (Figure 2D and Figure 3C); with the exception of sporadic staining of these cells for RPS4X/Y1, endothelial cells generally lacked the other mHAg proteins investigated in this study.

Maternal Tissues

The expression of mHAg in the decidua was determined by examining term basal plate and extraplacental membranes with their associated decidual tissue. HMHA1, RPS4X/Y1, and KIAA0020 were expressed by decidual cells, whereas BCL2A1 expression was weak or absent (Figure 3, B, D, F, H, and J). KIAA0020, BCL2A1, and RPS4X/Y1 were generally negative in maternal leukocytes associated with decidual tissue. Maternal leukocytes consistently expressed HMHA1 in both first- and third-trimester tissues. These cells could be observed both in peripheral blood located within the intervillous space and within decidual tissue associated with the chorion and basal plate.

Proteomic Analysis of the Syncytiotrophoblast and Trophoblast Debris

Proteomic analysis was performed on the syncytiotrophoblast and on the trophoblast debris shed from cultured first-trimester placental explants. A total of 1590 proteins were identified as being present in the syncytiotrophoblast, and 375 proteins were identified as being expressed in trophoblast debris, with a false-discovery rate of 0.0087. The identity of these proteins was confirmed by the characterization of at least two peptides from the protein sequences. Two autosomally encoded mHAg (solute carrier 1A5 and signal sequence receptor 1) and two Y chromosome-encoded mHAg (RPS4Y1 and DDX3Y) were found in the proteomic analysis. RPS4Y1, solute carrier 1A5, and signal sequence receptor 1 were identified in the syncytiotrophoblast, whereas DDX3Y was present in both the syncytiotrophoblast and the shed trophoblast debris (Table 2).

Discussion

Multiparous women have previously been shown to possess expanded cohorts of T cells specific for fetal

mHAg.^{13–15} However, the source of the antigens that trigger this T-cell expansion has not been definitively identified. In this study, we identified the placenta, a fetal organ, as a probable source of foreign mHAg to which the mother is exposed during pregnancy. By using RT-PCR, we demonstrated that mRNAs for the autosomal mHAg (HMHA1, KIAA0020, and BCL2A1) and the Y chromosome-encoded mHAg (KDM5D, DDX3Y, and RPS4Y1) are found in placental tissue and purified trophoblast cells. These results are consistent with those of Warren et al⁵⁶ who, by tissue array, identified mRNA for another male mHAg, UTY, in human placental homogenate. IHC analysis revealed that four of the six mHAg studied were expressed by several different placental cell types, including trophoblast subpopulations, mesenchymal cells, placental macrophages, and cord blood leukocytes. Importantly, all four mHAg examined by IHC were expressed within the syncytiotrophoblast, which, for most of the pregnancy, is bathed directly in maternal blood. This then provides an open avenue for release of mHAg-containing shed material from the placenta into the blood and subsequent trafficking to maternal lymphoid organs, where an immune response might be generated. Indeed, we show that at least one antigen, DDX3Y, is present in trophoblast debris shed from first-trimester placenta.

Our findings of robust expression of fetal mHAg in the placenta align with earlier work^{13–15,57} demonstrating that pregnancy results in the expansion of maternal minor antigen-specific CD8⁺ T cells. One antigen that elicits antigen-specific maternal immunoreactivity, HMHA1,¹⁶ was strongly expressed in the syncytiotrophoblast and EVT, both of which are fetal cells to which the maternal immune system has direct contact. Protein expression studies of other Y chromosome-encoded antigens known to elicit maternal T-cell reactivity, such as KDM5D,^{13,15} are limited by antibody availability and specificity. Indeed, caution in interpretation of RPS4Y1 IHC studies is warranted because the peptide immunogen for the RPS4X/Y1 antibody derives from regions conserved between the male and female isoforms of this protein, thus rendering the antibody undiscerning between male and female placentas. Nonetheless, we have shown the presence of the male-specific isoform, RPS4Y1, by both RT-PCR and proteomic analyses in the trophoblast of male placentas.

The finding that mHAGs are expressed in the syncytiotrophoblast and in trophoblast debris shed from the placenta provides the first direct evidence that trophoblast and deported trophoblast debris contain proteins that are responsible for triggering cell-mediated immune responses. A single mHAG was found in proteomic analysis of trophoblast debris shed from the placenta. It seems likely that other mHAGs are also present in the debris, because MS proteomic analysis is only able to detect proteins that are present in relatively high abundance; some of the mHAGs may be expressed at a level too low to detect by this method. Current efforts are focused on identifying these antigens in trophoblast debris using more sensitive methods.

Much trophoblast debris is thought to be shed into the maternal blood daily in a normal pregnancy.^{29,30} This enormous amount of trophoblast debris is rapidly cleared from the maternal blood by an unknown mechanism, but it is likely that maternal phagocytes, such as macrophages, are at least partially responsible. Phagocytosis of mHAG-containing trophoblast debris by maternal antigen-presenting cells would likely produce some form of maternal adaptive immune response. Additionally, the context in which the adaptive immune system detects antigen is critical in determining the nature of the ensuing response. Antigens produce tolerogenic immune responses when they are presented from apoptotic cells.⁵⁸ Because trophoblast debris is thought to be shed from the placenta via apoptosis,^{28,52} it seems likely that DDX3Y and other mHAGs and/or HLAs³⁶ in deported trophoblast debris would lead to a tolerogenic immune response that may be important in promoting the survival of placental allografts that express this protein. On the other hand, an excessive antigenic load and the association of antigen with necrotic cellular debris can incite inflammatory responses. The quantity of shed trophoblast cells and microparticles is increased in preeclamptic pregnancies,^{33,34} and reduced/fluctuating oxygen concentrations associated with preeclampsia may drive syncytial fragments in the process of deportation toward a necrotic, rather than an apoptotic, phenotype.⁵⁹ It follows, then, that the presence of mHAGs in high amounts of necrotic trophoblast debris may stimulate an active immune response to the placenta/fetus, which might occur in some cases of recurrent miscarriage or other pathological conditions of pregnancy.

An important consideration is the mechanism by which antigens derived from trafficking placental cells or debris might be presented by antigen-presenting cells. Little is known about the form in which fetal antigen may promote maternal lymphocyte expansion. In a mouse model of paternally inherited fetal antigen, the indirect pathway of antigen presentation occurs whereby maternal (but not fetal) antigen-presenting cells cross present fetal antigen to MHC class I-restricted maternal CD8⁺ T cells.^{10,11} Cross presentation can result in tolerance or priming of T cells, and occurs as a result of phagocytosis of apoptotic or necrotic cellular material by dendritic cells, with antigen processing occurring either before or after transfer to the recipient antigen-presenting cell.⁶⁰ Phagocytosis of apoptotic and necrotic human-deported trophoblasts by

macrophages has been recapitulated *in vitro*.²⁵ Intriguingly, the physiological state of the phagocytosed trophoblast material had a strong influence on the phenotype of the antigen-presenting cell; this may, in turn, translate into different functional outcomes of cognate interaction with lymphocytes. Such outcomes and their consequences in human pregnancy remain to be investigated.

The lymphocyte responses arising from fetal mHAGs may include generation of fetal antigen-specific regulatory T cells. van Halteren et al¹⁶ demonstrated the presence of putative regulatory CD8⁺ T cells in parous women, including those specific for SMCY and HMHA1, antigens that were identified in trophoblasts in this study. Interestingly, results using the abortion model of mating CBA/J female mice to DBA/2J males suggested that paternal minor antigen-specific CD4⁺ regulatory T cells could be protective against maternal anti-fetal responses.⁴ Thus, it is possible that antigen-specific regulatory-type cells are critical for conferring tolerance to these antigens.

It is likely that tolerance to paternally inherited antigens is induced at the time of first exposure to that antigen. From this study, however, it is not possible to discern the point at which women are first introduced to each mHAG. Studies^{5,11,61} in mice using model antigens suggest that if antigen is present, tolerance to that antigen can be established as early as insemination and, furthermore, that this early exposure is critical to pregnancy success. KDM5D protein on approximately 50% of ejaculated sperm has been reported,⁶² raising the possibility that this antigen is introduced to the maternal immune system at copulation and that tolerance to KDM5D could be established at that point. On the other hand, neither DDX3Y nor RPS4Y1 could be found on spermatozoa, despite their expression at the level of mRNA⁶²; it remains possible that these antigens are present in seminal fluid or, alternatively, that tolerance to these antigens occurs at some later time point. Other work^{63–65} suggested that undefined Y-encoded mHAGs are expressed on male preimplantation embryos of several species, although expression decreased at the blastocyst stage and was absent on trophectoderm. However, because of the lack of lymphatic drainage of the human decidua,⁶⁶ mHAG expression on preimplantation or early postimplantation embryos may go undetected. Taken together, the initial events of antigenic exposure and tolerance induction likely vary according to the spatial and temporal regulation of individual antigens. The route by which paternally inherited antigen is exposed to maternal lymphoid organs probably also depends on when and where the antigen is expressed. If first expression is on the placenta itself, the route is likely hematogenous; if onset of expression occurs in seminal fluid or on spermatozoa, exposure likely occurs via lymphatic drainage.

Another mechanism distinct from trophoblast shedding that could lead to maternal alloimmunization to fetal antigen is microchimerism. Herein, fetal progenitor cells actively traffic into the maternal blood and tissues, where they can be harbored for decades after childbirth.²³ Blood cells from the fetus may be the source of these cells,^{20,21,67,68} although the mechanism by which fetal

cells traffic into the maternal circulation is unknown. The presence of detectable mHAGs within fetal leukocytes is suggestive that microchimerism can be a mechanism of maternal alloimmunization to these antigens, as has been suggested by others.¹⁶ Indeed, some mothers possessing T cells directed against their children's mHAGs have possessed significant fetal microchimerism.^{14,21} Yet, another potential mechanism that could result in maternal exposure to fetal blood antigens is leakage into the maternal circulation during placental hemorrhage before or during parturition. Indeed, this occurs with isoimmunization of rhesus antigen-negative women carrying rhesus-positive fetuses.

Maternal alloimmunization by fetal antigens during pregnancy is thought to have important clinical implications for organ and stem cell transplantation, subsequent pregnancies, and autoimmunity. Donor parity has been identified as a risk factor for acute and chronic graft-versus-host disease in patients undergoing hematopoietic stem cell transplantation,^{17,18} and recipients of parous female hematopoietic stem cell transplantation have both higher rates of graft-versus-host disease and graft-versus-leukemia effects.^{6,69–71} Researchers hypothesize that mHAG-specific T cells, which had resulted from exposure of fetal mHAGs to the maternal compartment during pregnancy, are provided by the female donor. The long-term impact of pregnancy-induced maternal sensitization is demonstrated by the findings that mHAG-specific T cells may persist up to 22 years after pregnancy.^{6,15,72}

Secondary recurrent miscarriage is defined as three or more consecutive miscarriages after a birth at 24 weeks' gestation or later. Two independent research groups have found a significant association between the development of secondary recurrent miscarriage and prior birth of a male infant. This may be due, at least in part, to an aberrant maternal immune response to fetal Y chromosome-encoded antigens.^{73,74} In addition, maternal carriage of HY-restricting MHC class II alleles is associated with poor pregnancy outcomes in women with a prior live birth of a male infant and a diagnosis of secondary recurrent miscarriage.⁷⁵ Our data indicate that maternal exposure to Y chromosome-encoded fetal antigens is likely to occur during pregnancy, at least in part as a result of shedding and deportation of trophoblast debris into the maternal blood. Therefore, aberrant responses to mHAGs may contribute to inappropriate adaptive maternal immune responses to the placenta/fetus.

Finally, our results have implications for the relationship between fetal microchimerism and maternal autoimmune disease, in that they further the possibility of differential antigen recognition by the maternal immune system and/or engrafted fetal cells. Nelson et al⁷⁶ and other researchers^{77–79} noticed an increase in the prevalence of male DNA of fetal origin within blood and skin lesions of women with systemic sclerosis. The relationship between microchimerism and systemic sclerosis has not been definitively elucidated, but possibilities include mutual recognition of minor and major histocompatibility antigens by maternal cells or engrafted fetal cells.^{80,81} Because placental deportation may be a

mechanism of priming toward fetal antigen, the environmental context during pregnancy may determine future reactivity toward antigen on re-exposure in various states, including reactivity to microchimeric fetal cells, future pregnancy, and transplanted organs and cells.

In conclusion, we have demonstrated that paternally inherited fetal mHAGs are expressed in the fetal blood, the syncytiotrophoblast of the placenta, and trophoblast debris shed from the placenta. This finding provides support for the hypothesis that mHAG-specific T cells are generated during pregnancy as the result of both trophoblast deportation from the placenta into the maternal blood and fetal microchimerism. Importantly, these results refute the concept that the trophoblast is antigenically inert. Rather, the trophoblast is a source of fetal antigen that is likely to tolerize the maternal adaptive immune system in normal pregnancy, and may stimulate adverse responses by maternal immune cells in diseases of pregnancy and complications of transplantation.

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